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Original Article

Effects of human urine-derived stem cells on the cementogenic differentiation of indirectly-cocultured periodontal ligament stem cells

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Abstract: Human periodontal ligament stem cells (PDLSCs) have been widely applied as seed cells and cell sheets in periodontal tissue regeneration. Despite significant progress in PDLSCs application, it is a major challenge to promote cell proliferation and multiple differentiations of PDLSCs because cell numbers at the initial obtaining are limited. The goal of study was to determine the paracrine effects of human urine-derived stem cells (USCs) on cell proliferation and osteogenic differentiation of PDLSCs when USCs were indirectly-co-cultured with PDLSCs. After indirectly-co-cultured with USCs at different ratios (PDLSC/USC, 1/0.5, 1/1 and 1/2), number of PDLSCs among the three co-cultured groups visibly increased from day 5 to a similar extent, and the expression of osteogenic and cementogenic genes and proteins in the osteogenic medium significantly increased with an increasing proportion of USCs compared to USC-free control group. In addition, osteogenic matrix PDLSC sheets at a PDLSC/USC ratio of 1/2 contained denser collagen layers and exhibited increased osteogenic and cementogenic protein expression. In vivo transplantation showed that PDLSC sheets noncontact cocultured at a PDLSC/USC ratio of 1/2 formed more new and dense structures and expressed higher levels of osteogenic and cementogenic proteins. In conclusion, the present results demonstrate that USCs promote the proliferation and osteogenic and cementogenic differentiation of PDLSCs in a ratio-dependent manner through noncontact coculture and further accelerate the regeneration of new structures by osteogenic matrix PDLSC sheets in vivo. These results suggest their use as a new strategy for application in clinical periodontal tissue repair.

Keywords: Tissue engineering, periodontal ligament stem cells, urine-derived stem cells, cell sheet, periodontal regeneration

Introduction

Periodontitis, a chronic multifactorial disease that leads to the destruction of periodontal tissues, including the alveolar bone, periodontal ligament (PDL) and cementum, affects up to 90% of the population worldwide [1, 2]. Available clinical treatments for periodontitis, such as periodontal flap operation, guided tissue regeneration, graft transplantation and recombinant growth factor application, can be used either alone or in combination and can control inflammation and repair component periodontal tissue to a certain degree [3-5]. However, the ability of current clinical treatments to regenerate complete and functional periodontal tissue is limited [4-6]. Fortunately, there is a recent growing focus on stem cell-based tissue engineering as a novel treatment option for periodontal tissue regeneration [7, 8]. To date, the results obtained in both animal experiments and clinical studies using stem cells derived from various tissues have shown that the application of stem cells may be an effective way to repair periodontal defects [7-14].

Since stem cells in human PDL with multiple differentiation capability, generally termed periodontal ligament stem cells (PDLSCs), were iso-

lated in 2004, a great deal of evidence has indicated that these stem cells from the periodontium are more suitable as seed cells for periodontal cytotherapy than stem cells derived from other sources [13, 15-21]. Previous studies have shown that some trophic factors, such as vascular endothelial growth factor (VEGF), periostin (POSTN) and epidermal growth factor, have positive effects on the osteogenic differentiation of PDLSCs in vitro and the formation of mineralized structures in vivo [22-25]. Moreover, there is evidence that jaw bone marrow-derived mesenchymal stem cells, endothelial cells and osteoblast progenitors improve PDLSCs proliferation and differentiation through coculture [26-28]. However, the translation of biological factors into clinical treatments is suboptimal, the acquisition of such cells is invasive, and adverse side effects may exist [8, 29]. Human urine-derived stem cells (USCs) were first isolated from human urine in 2008; USCs are noninvasive, easy to collect, and stable in culture and have the potential for multidirectional differentiation [30-32]. USCs have been used as seed cells for various aspects of regenerative medicine, including bladder and urethral reconstruction, nerve regeneration and wound healing [33-36]. However, USCs exhibit a lower capacity for osteogenic and chondrogenic differentiation than bone marrow-derived mesenchymal stem cells and adipose tissue stem cells [31, 37]. Through paracrine effects, USCs efficiently attenuate severe hind-limb ischemic injury and particularly strengthen the chondrogenic differentiation of bone marrow stromal cells (BMSCs) after passaging in vitro through a Wnt11-mediated noncanonical signaling pathway by releasing trophic factors [38, 39]. The trophic factors of USCs include a variety of growth, inflammatory, and immunomodulatory factors [38]. Given the above data, we wanted to investigate the paracrine effect of USCs on PDLSCs through noncontact coculture.

In addition to considerations of seed cells, the delivery strategy for seed cells also needs to emphasize in the cell-based periodontal regenerative medicine [40]. A sufficient number of vital seed cells in the defect area must first be ensured in routinely applied clinical therapies [41, 42]. To this end, scaffold-free cell sheet technology allows for the delivery of a complete sheet of interconnected cells that rely on induced extracellular matrix (ECM) secretion [43]. Although cell sheet technology has been widely used for seed cell delivery to the periodontium, concerted efforts are still being made to harvest living cell sheets more easily and effectively [43]. Currently, vitamin C (Vc) and osteogenic induction are practical ways to harvest cell sheets, and osteogenic matrix cell sheets have been widely used in bone tissue regeneration [44-46]. Thus, we wanted to investigate the biological effect of USCs on the cell sheet formation and osteogenic/cementogenic properties of PDLSCs.

Based on the previous research results detailed above, we hypothesized that the paracrine effect of USCs enhances the differentiation and proliferation of PDLSCs and the formation of PDLSC sheets at the optimal ratio to carry out these desired functions. To apply PDLSC sheets formed by noncontact coculture with USCs in clinical situations, it is important to determine the influence of USCs on the generation of periodontal structures by PDLSCs. Thus, Transwell chambers were used to build a noncontact coculture system in vitro, and nanocrystalline hydroxyapatite (HA) with osteogenic matrix PDLSC sheets noncontact cocultured with USCs was implanted subcutaneously in nude mice to investigate the generation of periodontal-like structures in vivo.

Materials and methods

The present work was developed according to the principles recommended for experimentation with human beings and animals determined by the Institutional Review Board of the Stomatological Hospital of Chongqing Medical University, and ethics committee approval was obtained (No. 2019-024). All subjects enrolled were informed about the procedures and objectives of the study and signed a consent form.

Cell isolation and culture

PDLSCs were isolated from healthy PDL tissues of premolars extracted from donors (ten donors aged 12-18 years) undergoing orthodontic treatment. Primary PDLSC isolation and proliferation were performed as described previously with minor changes [21]. Briefly, the extracted teeth were washed twice in sterile phosphate-buffered saline (PBS), and then PDL tissues were gently removed with a scalpel

from the middle third of the root surface and digested with 3 mg/ml collagenase type 1 (COL-1, Sigma-Aldrich, USA) for 30 min at 37°C. After digestion, tissues were seeded into cell culture flasks (Corning, Lowell, MA, USA) with α-minimum essential medium (α-MEM, Sigma-Aldrich, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, BRL, USA) and then incubated in 5% CO_2 at 37°C until cells had grown out from tissues and approached confluence. USCs were isolated from human urine samples (100-400 ml/sample) collected from healthy volunteers (22-26-year-old males). USC isolation and proliferation were performed as described previously [30]. In brief, fresh urine samples containing penicillin and streptomycin were transferred immediately to the laboratory for centrifugation at 500 × g for 5 min at room temperature; then, the supernatant was removed, and the cell pellet was gently resuspended in USC-specific medium. The main USC medium was a blend of keratinocyte serum-free medium (Gibco, BRL, USA) and progenitor cell medium at a 1:1 ratio. Progenitor cell medium contained 1/4 Ham's F12 medium (Sigma-Aldrich, USA), 10 % FBS, 3/4 Dulbecco's modified Eagle's medium (Sigma-Aldrich, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, USA). Other supplements in the USC-specific medium were epidermal growth factor (EGF, Gibco, BRL, USA), hydrocortisone, bovine pituitary extract (BPE, Gibco, BRL, USA), L-glutamine (Sigma-Aldrich, USA), triiodo-L-thyronine (Sigma-Aldrich, USA), insulin (Gibco, BRL, USA), transferrin, and adenine (Sigma-Aldrich, USA). After adequate mixing, a single cell suspension was plated in 24-well plates and incubated in 5% CO_2 at 37°C. The USC medium was first changed after 4 days of culture and refreshed twice a week thereafter. When USCs reached 70-80% confluence in 24-well plates, they were passaged by digestion with 0.25% trypsin containing 1 mM EDTA and transferred to 100-mm dishes for expansion. For most experiments, USCs and PDLSCs at passage 3-5 were used.

Colony formation assays

Single cell suspensions containing 300 PDLSCs or USCs were seeded in 6-well culture plates and cultured with the respective basal medium to assess cell proliferation. The cells were fixed with 4% paraformaldehyde and stained with a 1% toluidine blue solution (Solarbio, China) after 10 days of culture. Aggregates of 50 or more cells were counted as colonies under a microscope (Nikon, Tokyo, Japan), and the numbers of colonies per well were counted.

Osteogenic/adipogenic differentiation

A total of 1×10^5 PDLSCs or USCs were seeded in six-well plates and cultured with the respective basal medium. Adipogenic medium (10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 mM hydrocortisone, and 100 mg/l indomethacin) was used for cells at 100% confluence, and osteogenic medium (10% FBS, 100 mM dexamethasone, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate) was used for cells at 80% confluence. The induction medium was changed every 2-3 days. Then, the cells were fixed with 4% paraformaldehyde for 20 min after 3 weeks of culture. Mineral deposit formation was identified by 2% alizarin red (Solarbio, China) staining, and lipid droplet formation was determined by Oil Red O (Solarbio, China) staining at room temperature. Finally, the cells were routinely observed and photographed under an inverted microscope.

Flow cytometric analysis of cell phenotypes

To identify cell phenotypes, flow cytometry was used to measure the cell surface marker expression of PDLSCs and USCs at passage 3. A single cell suspension containing approximately 1×10^6 cells was resuspended in PBS containing 2% FBS. To assess the mesenchymal stem cell (MSC) phenotype, cells were incubated with FITC-conjugated monoclonal antibodies against human CD31, CD34, CD45, CD90 and CD105 (BD Pharmingen™) for 30 min on ice. Cells incubated with isotype controls for the primary antibodies were used as negative controls. Then, the cells were washed twice to remove unbound antibody in the dark. Finally, surface antigen analysis was performed using a flow cytometer (BD Influx™).

Group design

To examine the effect of USCs on PDLSCs, Transwell chambers (Millipore, USA) with 0.4-μm membrane pores were used to set up a noncontact coculture system [26]. To assess proliferation, PDLSCs and USCs were noncontact cocultured at 3 ratios in 24-well plates as follows: group 1: 1:0.5 (4×10^3 PDLSCs and 2×10^3 USCs), group 2: 1:1 (4×10^3 PDLSCs and 4×10^3 USCs), group 3: 1:2 (4×10^3 PDLSCs and

Table 1. Primer sequences

Gene	Forward	Reverse
ALP	5'-TGGCAGTGTCCAGGGAAGAA-3'	5'-AACGCAGGATTTCCCACACTA-3'
RUNX2	5'-CACTGGCGCTGCAACAAGA-3'	5'-CACTGGCGCTGCAACAAGA-3'
OCN	5'-CCCAGGCGCTACCTGTATCAA-3'	5'-GGTCAGCCAACTCGTCACAGTC-3'
POSTN	5'-ACTTTGCTGGCACCTGTGAATA-3'	5'-TCCGATGGTTTCCAGATTTTGC-3'
CEMP1	5'-ACATTTCCTTAACTGGGCTTTGC-3'	5'-ACATTTCCTTAACTGGGCTTTGC-3'
β-ACTIN	5'-CCACGAAACTACCTTCAACTCC-3'	5'-GTGATCTCCTTCTGCATCCTGT-3'

8×10^3 USCs). As a control, 4×10^3 PDLSCs were also cultured in a monolayer in USC-free media. To assess differentiation, PDLSCs and USCs were cocultured while segregated at the same ratios used to assess proliferation in 6-well plates as follows: group 1: 1:0.5 (1×10^5 PDLSCs and 0.5×10^5 USCs), group 2: 1:1 (1×10^5 PDLSCs and 1×10^5 USCs), group 3: 1:2 (1×10^5 PDLSCs and 2×10^5 USCs). As a control, 1×10^5 PDLSCs were also cultured in a monolayer in osteogenic medium. For all of the above groups, PDLSCs were cultured on the bottom of the culture plate (lower well), and USCs were cultured on the Transwell insert (upper well).

Cell proliferation assay

The influence of USCs on the proliferation of PDLSCs was analyzed by CCK-8 assays (Dojindo Laboratories, Japan). According to the manufacturer's instructions, 1/10 CCK-8 solution in culture medium was added to each well, and the plates were then incubated at 37°C for 2 h at 1, 3, 5 and 7 days after plating. The absorbance at 450 nm was measured using a microplate reader (EnSpire, USA).

Alkaline phosphatase (ALP) staining and activity

To stain for ALP, cocultured cells were treated with osteogenic medium for 7 days. PDLSCs were washed twice with PBS after fixation in 4% paraformaldehyde for 20 min at 4°C. ALP staining and activity were examined with a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China) and Alkaline Phosphatase (AKP/ALP) Detection Kit (Beyotime, China) according to the manufacturer's suggested protocols.

Alizarin red staining and calcium quantification

To observe calcium deposition in the ECM, cocultured cells were treated with osteogenic medium for 21 days. PDLSCs were washed twice with PBS after fixation in 4% paraformaldehyde for 20 min at room temperature. Calcium accumulation was detected by 2% alizarin red staining, and images were captured under an inverted microscope. To quantify the degree of mineralization, accumulated calcium was dissolved in 1 mL of a sodium dodecyl sulfate solution (Solarbio, Beijing, China), and the absorbance at 562 nm was measured using a microplate reader.

Real-time polymerase chain reaction (RT-PCR)

To measure the gene expression of ALP, runt-related transcription factor-2 (RUNX2), osteocalcin (OCN), POSTN and cementum protein 1 (CEMP1), cocultured cells were treated with osteogenic induction medium for 7 days. Total RNA was extracted from PDLSCs with RNAiso Plus (TaKaRa Bio, Otsu, Japan) according to the manufacturer's protocol. Then, 1 μg of total RNA was converted to cDNA with a TaKaRa Prime-Script RT Reagent Kit (TaKaRa Bio, Otsu, Japan), and PCR was performed using TB Green Premix Ex Taq II (TaKaRa Bio, Otsu, Japan) and a quantitative PCR System (Bio-Rad, Hercules, CA, USA). The housekeeping gene β-actin was used as an internal control for PCR amplification. The primer pairs used to amplify the target genes are listed in **Table 1**.

Western blot (WB) assay

To quantify the protein expression of ALP, RUNX2, OCN, POSTN and CEMP1, cocultured cells were treated with osteogenic induction medium for 7 days. Briefly, total cellular protein was extracted from PDLSCs with ice-cold RIPA lysis buffer (Beyotime, China) with a protease inhibitor cocktail (Beyotime, China). The protein concentrations of the lysates were measured using a BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes of the appropriate size (GE Healthcare, UK). The membranes were blocked with 5% nonfat dry milk for 1 h and then incubated with primary antibodies over-

night at 4°C. Next, the membranes were incubated with a secondary antibody for 1 h. The targeted proteins were detected with the ECL Plus Western Blotting Detection System (GE Healthcare, UK). The antibodies used in this work were against ALP, OCN, CEMP1 (Abcam, Cambridge, UK), RUNX2 (Cell Signaling, USA), POSTN, and β-actin, and HRP-conjugated secondary antibodies were against rabbit and mouse (Bioss, Beijing, China).

Enzyme-linked immunosorbent assay (ELISA)

To measure the expression of VEGF and basic fibroblast growth factor (b-FGF) in medium of USCs and the noncontact coculture system, conditioned medium was collected and analyzed by ELISA (Jiyinmei, Wuhan, China) according to the manufacturer's instructions. A total of 1×10^5 USCs at passage 3 were seeded in a six-well plate and the medium was collected after incubation with serum-free α-MEM at 37°C in 5% CO_2 for 24 h or 48 h. In the noncontact coculture system, the old medium was collected when changing the medium during the period of noncontact coculture with general medium or osteogenic medium. Briefly, 10 μl of culture supernatant was added to the 96-well plates and combined with the specific antibody. Then, a horseradish peroxidase (HRP)-conjugated antibody was added to each well and incubated at 37°C for 30 min. The optical density (OD) was measured at a wavelength of 450 nm after specific visualization. The concentration in the samples was calculated by comparing the OD of the samples to the standard curve.

Construction of cell sheets

PDLSCs and USCs at the same ratios used to assess differentiation were used to construct cell sheets in 6-well plates as follows: group 1: 1:0.5 (1×10^5 PDLSCs and 0.5×10^5 USCs), group 2: 1:1 (1×10^5 PDLSCs and 1×10^5 USCs), group 3: 1:2 (1×10^5 PDLSCs and 2×10^5 USCs). As a control, 1×10^5 PDLSCs were also cultured in a monolayer in osteogenic medium. After PDLSCs reached 80% confluence, the noncontact cocultured cells were treated with osteogenic induction medium for 10 days. After 10 days, the cell sheets were harvested and easily detached from the culture plates with a cell scraper.

Observation by scanning electron microscopy (SEM)

The detached cell sheets from each group were washed with PBS three times and fixed with 2.5% glutaraldehyde at 4°C overnight. The samples were dehydrated, dried and anodized in an electrolyte solution containing 0.5 wt % hydrofluoric acid and 1 M phosphoric acid for 1 h. Then, the whole sample was observed by SEM (Hitachi SU-8010, Tokyo, Japan).

Hematoxylin and eosin (H&E) and immunohistochemical staining

The detached cell sheets were fixed in 4% paraformaldehyde for 2 h, paraffin-embedded and then cut into 5-μm thick sections for H&E staining. Other sections were subjected to immunohistochemical staining. Briefly, sections were deparaffinized, immersed in 3% H_2O_2/methanol and then incubated with an optimal concentration of anti-OCN (1:200, Abcam), anti-periostin (1:200, Bioss), and anti-CEMP1 (1:400, Bioss) primary antibodies overnight at 4°C. Next, the sections were incubated with secondary antibodies (1:1000, Bioss) and stained with 3, 3'-diaminobenzidine (DAB). The stained sections were then observed under an inverted microscope.

Ectopic transplantation into nude mice

Ten 6-week-old immunodeficient mice (BALB/c-nu; Chongqing Medical University Medical Laboratory Animal Center, Chongqing, China) were used in this experiment. Thirty milligrams of HA was wrapped into a ball surrounded by three-layered cell sheets, and the cell sheet graft complexes were then incubated with a small quantity of culture medium at 37°C for 1 h to allow better adhesion. Mice were anesthetized and transplanted with different grafts as follows: group 1 (30 mg HA with PDLSC sheets at a PDLSC/USC ratio of 1/2) and group 2 (30 mg HA with PDLSC sheets without USC treatment). Each mouse received two grafts, one on each side. The wounds were sutured to achieve primary closure. Six weeks after transplantation, the mice were euthanized, and the grafts were removed for histological analysis. All animal procedures followed the guidelines set forth by the Institutional Animal Care Committee.

Histological analysis

All specimens were fixed in 4% paraformaldehyde for 24 h, decalcified with 17% ethylenediaminetetraacetic acid (EDTA), and embedded in paraffin. One group of paraffinized sections (5-μm thick) was processed for H&E and Masson's trichrome staining according to the manufacturer's instructions. The other group of sections was used for immunohistochemical staining as described above. The antibodies used in this work included primary antibodies against OCN (1:200, Abcam), POSTN (1:200, Bioss) and CEMP1 (1:400, Bioss) and secondary antibodies (1:1000, Bioss). The stained sections were observed under an inverted microscope. The relative expression of target proteins was calculated from at least 3 randomly selected fields from each group using Image-Pro Plus 6.0 software.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Quantitative data were statistically assessed using an Student's t test when only two groups were compared and a one-way analysis of variance (ANOVA) when many groups were compared with SPSS version 23.0 software (SPSS, Inc., Chicago, IL, USA). *P* values less than 0.05 indicated statistical significance.

Results

Isolation and characterization of PDLSCs and USCs

Primary PDLSCs/USCs were successfully obtained from PDL tissues and urine and maintained a stable morphology after passage. Primary PDLSCs were observed around tissue pieces and exhibited a fibroblast-like morphology after passage (**Figure 1A**, **1B**). The primary USCs were observed in culture plates approximately 3 to 7 days after initial seeding, after which they reached confluence after 12 days and exhibited an elongated shape after passage (**Figure 1H**, **1I**). Both types of cells formed colonies and the percentages of PDLSCs/USCs that formed colonies were 28.93% ± 1.41 and 22.4% ± 1.27, respectively (**Figure 1C**, **1D**, **1J**, **1K**). After induction in osteogenic medium or adipogenic medium for 21 days, PDLSCs/USCs formed mineralized ECM, as observed by alizarin red staining (**Figure 1E**, **1L**), or accumulated lipid droplets, as observed by Oil Red O staining (**Figure 1F**, **1M**). In addition, flow cytometry demonstrated that the PDLSCs/USCs were positive for the MSC markers CD90 and CD105 and negative for the markers CD31, CD34 and CD45 (**Figure 1G**, **1N**).

Cellular effects of noncontact cocultured USCs on PDLSCs

The effects of USCs on PDLSC proliferation were determined by CCK-8 assays on days 1, 3, 5 and 7 (**Figure 2**). The number of PDLSCs cocultured with USCs was significantly higher after 5 days of culture than the number of PDLSCs cultured in monolayers without USCs. In addition, although PDLSCs/USCs at a ratio of 1/1 showed more active proliferation, there was no significant difference in active proliferation among the three groups cocultured with USCs. ALP staining and alizarin red staining showed that PDLSCs noncontact cocultured with USCs formed more mineral nodules and exhibited higher ALP activity than PDLSCs not cultured with USCs (**Figure 3A**). ALP activity and calcium content exhibited the same trend, and PDLSC osteogenesis increased with the ratio of USCs (**Figure 3B**, **3C**). To further analyze the effects of USCs coculture on PDLSCs, RT-PCR and WB analysis showed that PDLSCs cocultured with USCs had significantly higher gene and protein expression of the osteogenesis-related genes ALP, RUNX2, OCN, and POSTN than the corresponding controls (**Figure 4A-D**, **4F**, **4G**). In addition, expression of the cementogenesis-related gene CEMP1 at the gene and protein level was significantly higher in PDLSCs cocultured with USCs than in the corresponding controls and increased with the increasing proportion of USCs (**Figure 4E-G**).

Expression of VEGF and b-FGF in conditioned medium

The ELISA results suggested that the trophic factors secreted by USCs included VEGF and b-FGF. There was no significant difference in the expression of b-FGF in the supernatant of USCs at 24 h or 48 h. The expression of VEGF in the supernatant of USCs was slightly higher at 24 h than at 48 h (**Figure 5A**). The expression of VEGF and b-FGF was higher in general medium from PDLSCs cocultured with USCs than in that

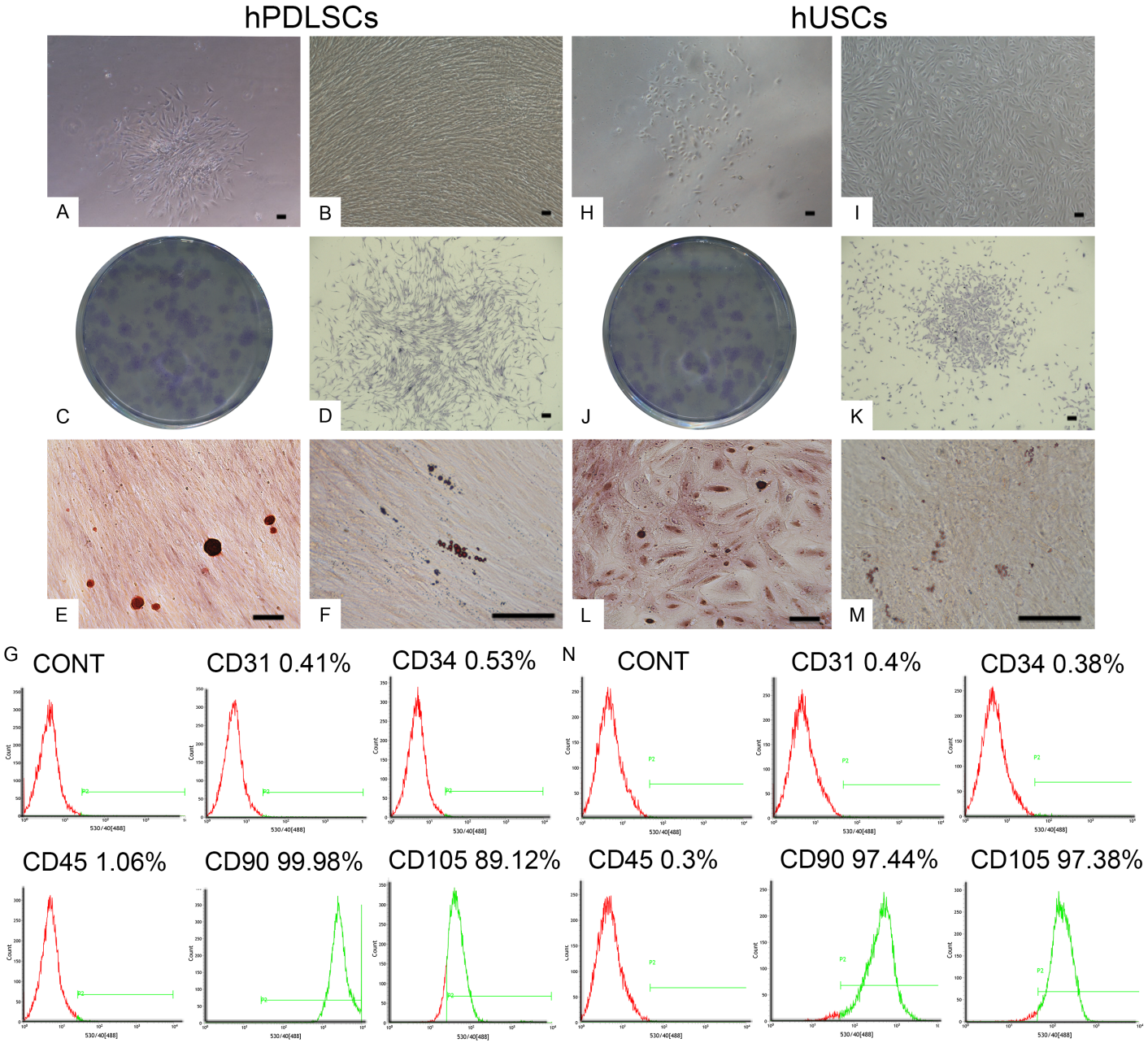
hPDLSCs
hUSCs
A
B
H
I
C
D
J
K
E
F
L
M
G
CONT
CD31 0.41%
CD34 0.53%
CD45 1.06%
CD90 99.98%
CD105 89.12%
N
CONT
CD31 0.4%
CD34 0.38%
CD45 0.3%
CD90 97.44%
CD105 97.38%

Figure 1. Isolation and characterization of PDLSCs /USCs. A, H. Primary human PDLSCs were observed around tissue pieces, and USCs were observed in culture plates. B, I. hPDLSCs exhibited a fibroblast-like morphology after passage, and hUSCs exhibited an elongated shape after passage. C, D, J, K. Representative figures show the proliferation of a single clone of hPDLSCs/hUSCs. E, L. Mineralized nodule formation following osteogenic induction is indicated by positive alizarin red S staining. F, M. Lipid droplets were found by Oil Red O staining following adipogenic induction. G, N. Flow cytometric analysis of exvivo-expanded hPDLSCs/hUSCs revealed positive CD105 and CD90 expression and negative CD31, CD34 and CD45 expression. The scale bar represents 50 μm. PDLSCs, human periodontal ligament stem cells; USCs, human urine-derived stem cells.

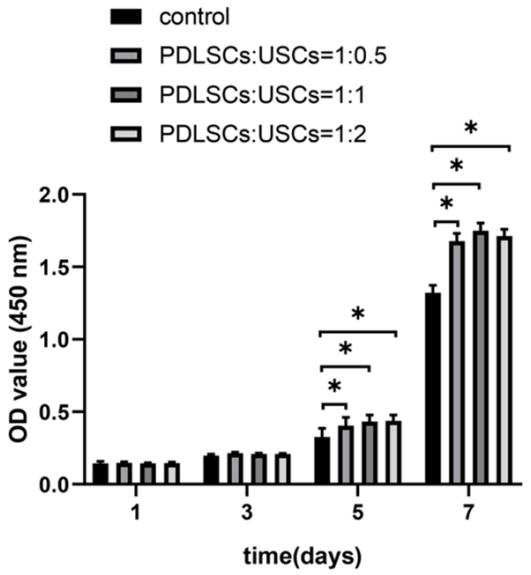

Figure 2. The effects of USCs on the proliferation of PDLSCs. The OD value was determined by CCK-8 assays on days 1, 3, 5 and 7. The proliferation activity of PDLSCs cocultured with USCs was significantly higher than that of PDLSCs cultured in monolayers without USCs after 5 days. The data are shown as the mean ± SD. *P < 0.05.

from PDLSCs cultured without USCs on day 3 to day 5 and day 5 to day 7 (**Figure 5B**). In osteogenic medium, the expression of VEGF and b-FGF in PDLSCs cocultured with USCs was significantly higher than in PDLSCs cultured without USCs (**Figure 5C**).

Effects of noncontact coculture with USCs on PDLSC sheets

After 10 days of culture, the four groups of PDLSCs all formed complete cell sheets that were segregated at the edge of the well plates. The cell sheets in all groups were ivory and exhibited a visible membrane-like morphology (**Figure 6A-D**). SEM demonstrated that the cell sheets from all four groups had a dense film-like cellular network that maintained tight junctions between the cells, and the PDLSC sheets cocultured with USCs contained denser collagen layers than the control PDLSC sheets (**Figure 6E-H**). As shown by H&E staining, the cell sheets from all four groups were dense and contained many cells. Furthermore, USCs coculture promoted the generation of more layers and more ECM by the cell sheets, and ECM generation in the PDLSC sheet was promoted to the greatest extent at a PDLSC/USC ratio of 1/2 was promoted to the greatest extent (**Figure 6I-L**). Immunohistochemical staining showed that the cell sheets of all four groups expressed OCN, POSTN and CEMP1 (**Figure 7A**), while the PDLSC sheets at a PDLSC/USC ratio of 1/2 expressed higher levels of all the target proteins than the corresponding controls (**Figure 7B-D**).

Regenerative hard tissue formation in vivo

At 6 weeks after cell sheet implantation into the subcutaneous space of immunodeficient mice, all samples were harvested and examined by H&E and Masson's trichrome staining. H&E and Masson's trichrome staining showed that a quantity of dense and notable collagenous fibers had formed in both groups, while regenerated tissues in the corresponding control group were few in number and scattered (**Figure 8A**, **8A1**, **8B**, **8B1**, **8C**, **8C1**, **8D**, **8D1**). The quantification of the percentage of the positive area to the total area showed the same trend (**Figure 8F**). Furthermore, immunohistochemical staining showed that the expression levels of CEMP1 and POSTN were higher in the experimental group than in the control groups, even though there was no difference in OCN expression between the two groups (**Figure 8E**, **8G**).

Discussion

The overall results of this study demonstrate that USCs at a proper ratio promote the proliferation and osteoblastic/cementoblastic differentiation of PDLSCs through noncontact cocul-

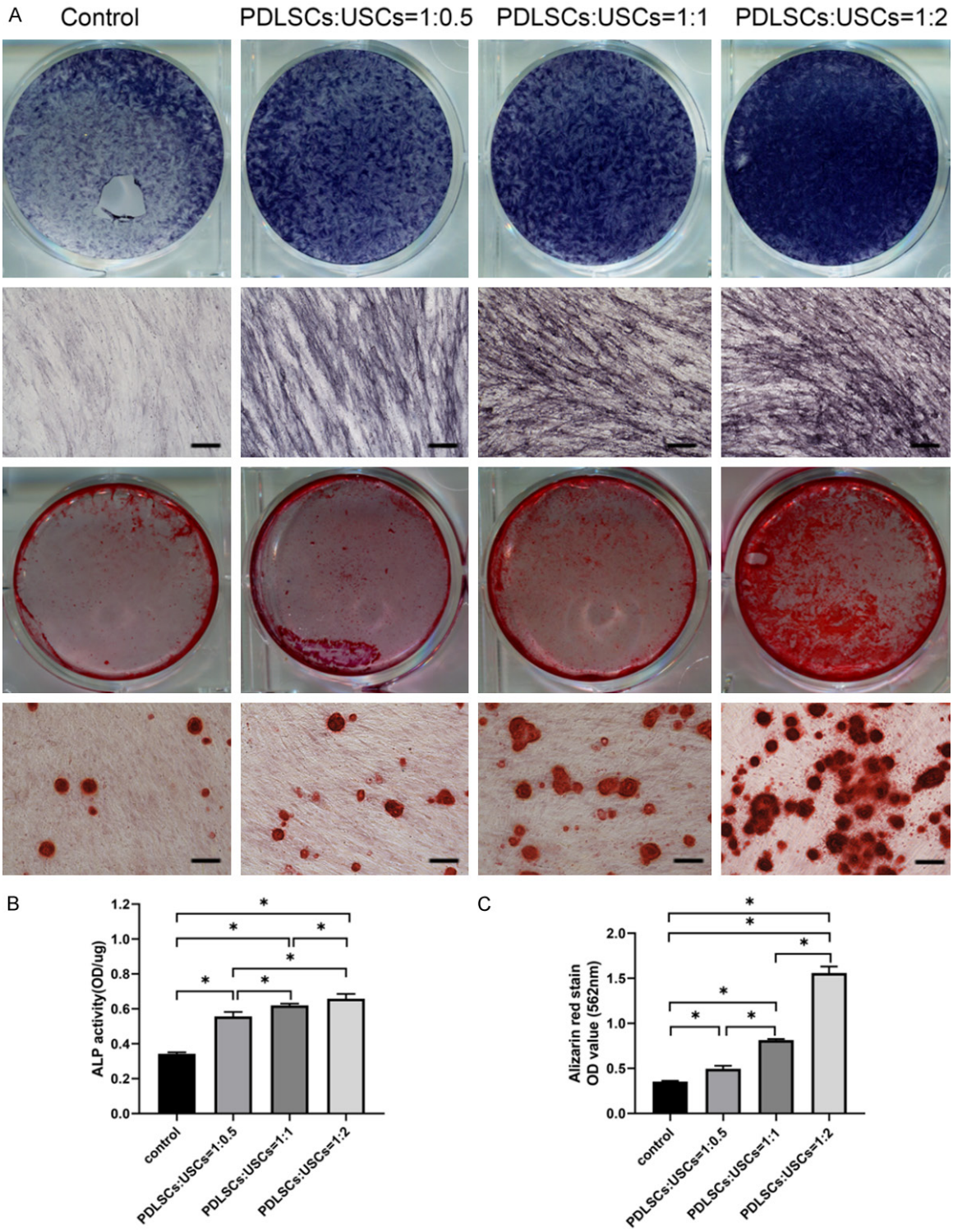

Figure 3. The effects of USCs at different ratios on the osteogenic differentiation of PDLSCs. A. Representative figures of ALP staining of PDLSCs treated with osteogenic medium for 7 days and alizarin red staining of PDLSCs treated with osteogenic medium for 21 days. B, C. The quantification of ALP activity and alizarin red staining showed the same trend in which PDLSCs noncontact cocultured with USCs exhibited higher ALP activity and formed more mineral nodules. The data are shown as the mean ± SD. *P < 0.05. The scale bar represents 50 μm.

ture and that this effect increased with the proportion of USCs. Furthermore, USCs were conducive to cell sheet formation and the osteoblastic/cementoblastic differentiation of

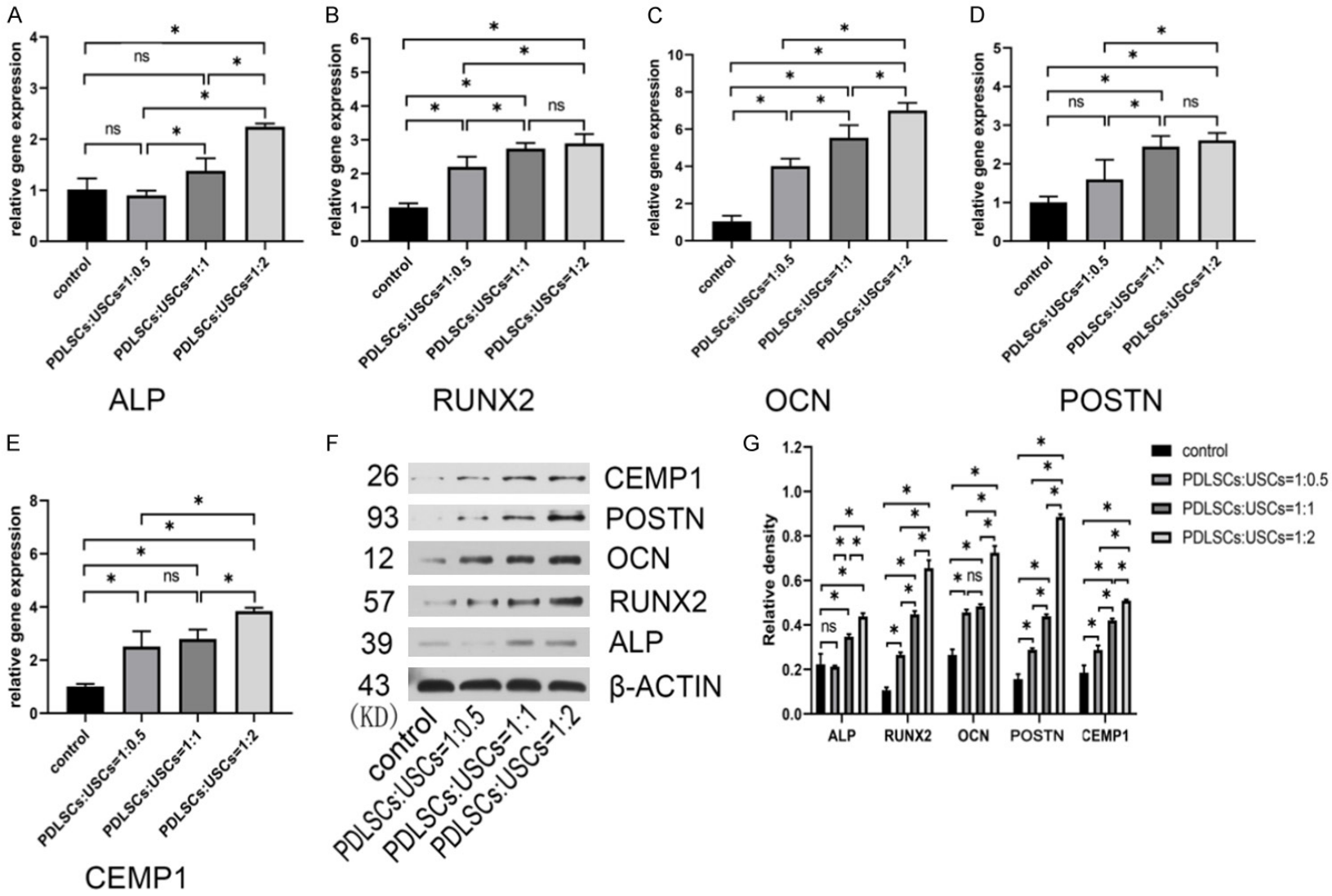

Figure 4. The effects of different ratios of USCs on PDLSC multiple differentiation. A-E. The PCR results showed that PDLSCs cocultured with USCs had significantly higher osteogenesis/cementogenesis-related genes expression than the corresponding controls after osteogenic induction for 7 days. F, G. The Western blot results showed that PDLSCs cocultured with USCs had significantly higher osteogenesis/cementogenesis-related proteins expression than the corresponding controls after osteogenic induction for 7 days. The data are shown as the mean ± SD. *P < 0.05.

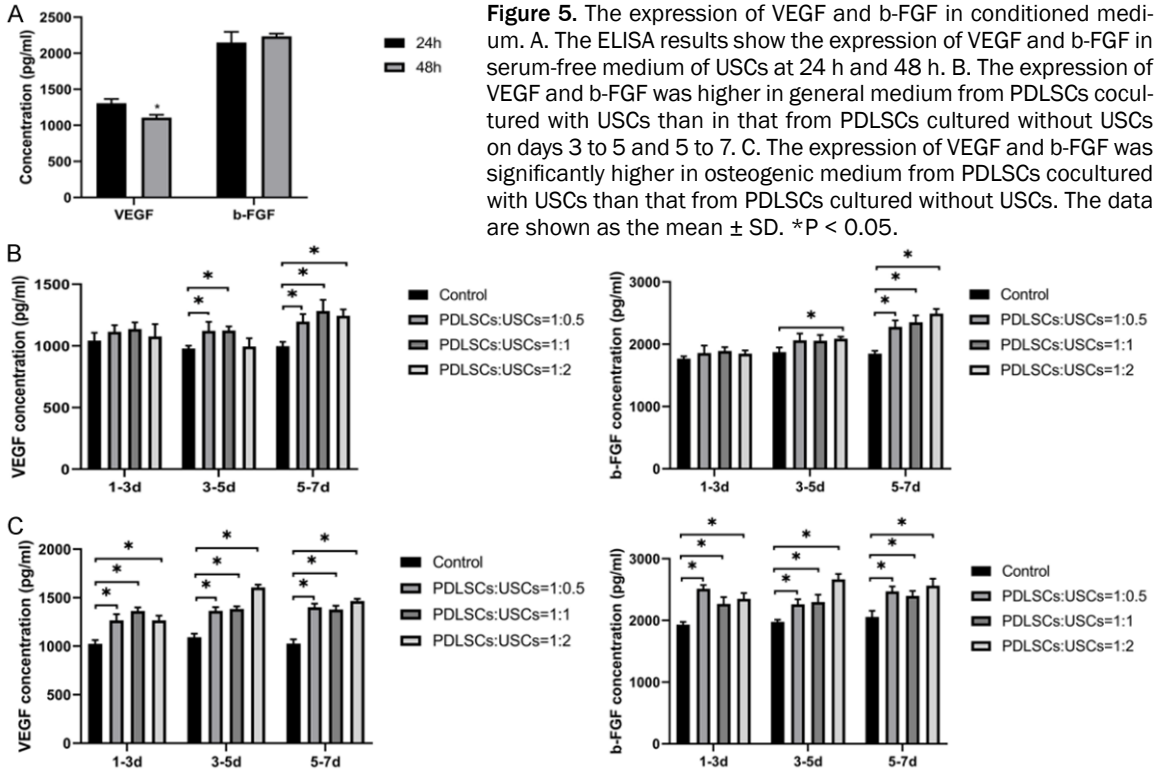

Figure 5. The expression of VEGF and b-FGF in conditioned medium. A. The ELISA results show the expression of VEGF and b-FGF in serum-free medium of USCs at 24 h and 48 h. B. The expression of VEGF and b-FGF was higher in general medium from PDLSCs cocultured with USCs than in that from PDLSCs cultured without USCs on days 3 to 5 and 5 to 7. C. The expression of VEGF and b-FGF was significantly higher in osteogenic medium from PDLSCs cocultured with USCs than that from PDLSCs cultured without USCs. The data are shown as the mean ± SD. *P < 0.05.

PDLSCs in vitro. The PDLSC sheets cocultured with USCs in combination with nanocrystalline HA exhibited increased osteoblastic/cementoblastic differentiation in vivo compared with the control PDLSC sheets. In this study, the paracrine effect of USCs enhanced the proliferation and differentiation of PDLSCs and may therefore be an alternative choice for periodontal tissue regeneration.

Recently, to overcome the limitations of traditional clinical periodontal treatments used to regenerate periodontal tissue, periodontal tissue engineering based on the use of stem cells has received widespread attention and has undergone thorough investigation. In particular, it is necessary to maintain the complete structure of bone, PDL fibers and cementum during the regeneration process during periodontal tissue engineering [20]. Cementum is a connective tissue covering the roots of teeth that connects with the PDL and alveolar bone to form a functional structure. Therefore, cementogenesis is a key process for periodontium regeneration [47]. CEMP1 expression was shown to be limited to cementoblasts, PDL cells and cells around the vessels in periodontium cementoblasts, indicating that CEMP1 is a marker protein for cementoblasts and their progenitor cells [48]. Although the addition of growth factors, such as platelet-derived growth factor, insulin-like growth factors and enamel proteins, can induce the formation of cementum/bone-like structures for periodontal regeneration, the application of biological factors is suboptimal because of the decrease in topical factors over time [49-52]. USCs, which can be harvested from voided urine via an easy, safe, low-cost and noninvasive method, possess stem cell characteristics and have high proliferative capacity and multipotent differentiation potential [30, 37, 38]. o date, research on the application of USCs to periodontal tissue engineering has not been conducted. Our results demonstrate that USCs can promote CEMP1 expression in PDLSCs in not only an in vitro coculture system but also in tissue regenerated in vivo after osteogenic induction; the use of USCs may be a new strategy to regenerate cementum for periodontal tissue engineering. A previous study showed that osteogenic stimulation inhibits the cementogenic pathways while upregulating the osteogenic pathways of PDLSCs, whereas Vc induction promoted the cementogenesis of PDLSCs for periodontal regeneration [53]. In our research,

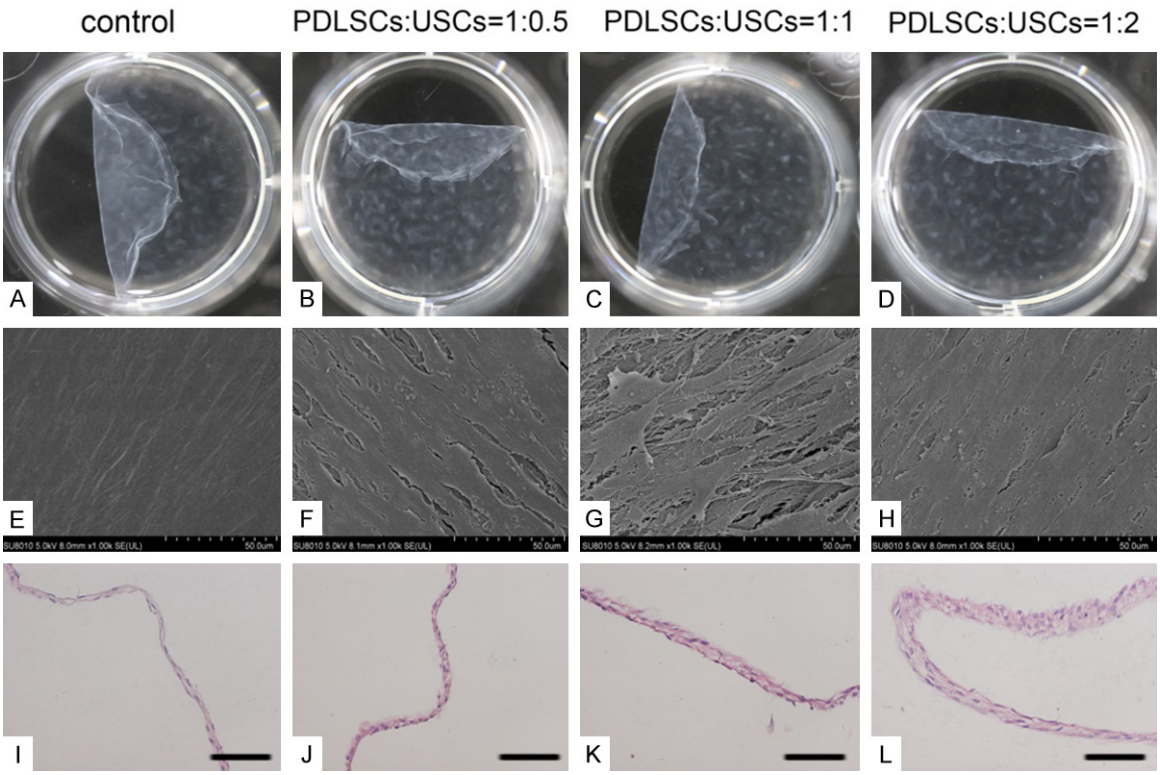

Figure 6. Morphological characteristics of noncontact PDLSC sheets cocultured with USCs. A-D. Representative macroscopic images of PDLSC sheets formed in culture plates after osteogenic induction medium for 10 days exhibited a visible membrane-like morphology. E-H. Representative SEM images of PDLSC sheets revealed a dense film-like cellular network that maintained tight junctions between the cells. I-L. Representative H&E staining images of PDLSC sheets showed dense ECM. The scale bar represents 50 μm.

USCs promoted the expression of both cementogenic and osteogenic genes through noncontact coculture in osteogenic medium, which indicated that two balanced antagonistic pathways may contribute to periodontal tissue regeneration.

In periodontal tissue regeneration, the microenvironment plays an important role in regulating seed cells [54]. Various cellular factors in the microenvironment, including autocrine and paracrine factors, can facilitate the regeneration of complex tissues by affecting the proliferation and differentiation of stem cells [55]. However, cellular factors secreted by a single cell type are limited and cannot adequately maintain diversity in the microenvironment [13]. Interestingly, previous studies have shown that USCs secrete a variety of trophic factors, including various types of growth, inflammatory, and immunomodulatory factors. Growth factors, especially VEGF-related factors, deserve more attention [38]. Therefore, we reconfirmed the content of VEGF and b-FGF in the serum-free medium of USCs. At the same time, VEGF and b-FGF content in the general medium or osteogenic medium of the coculture system was detected. Although there was serum interference, the results showed that USCs could increase the concentrations of VEGF and b-FGF in the noncontact coculture system. Previous studies demonstrated that VEGF and b-FGF at certain concentration ranges have a positive effect on proliferation, which is consistent with our observations that there was no difference in the proliferation of PDLSCs cocultured with USCs at three ratios. In addition, previous studies showed that VEGF can promote osteogenic differentiation, but exogenous b-FGF may slightly inhibit osteogenesis. When VEGF and b-FGF exist in a system, the synergistic effect was shown to depend on the proportion [22]. In our study, the expression of osteogenesis-related genes and proteins tended to increase with the ratio of USCs noncontact cocultured with PDLSCs. Therefore, USCs can promote the pro-

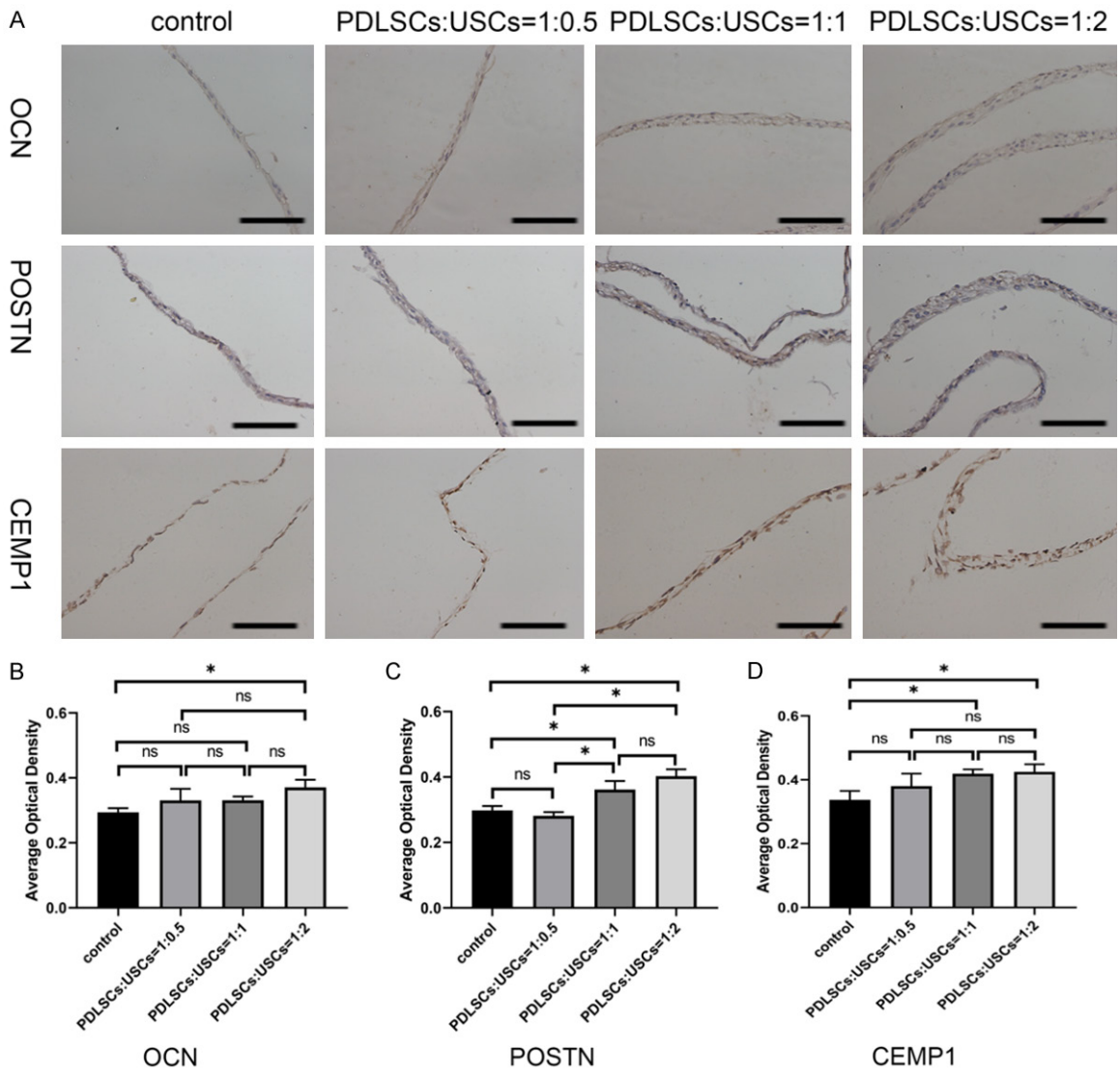

Figure 7. Immunohistochemical analysis of noncontact PDLSC sheets cocultured with USCs. A. All cell sheets positively expressed OCN, POSTN and CEMP1. B-D. The quantitation of OCN, POSTN and CEMP1 immunohistochemical staining showed that the PDLSC sheets at a PDLSC/USC ratio of 1/2 expressed higher levels of all the target proteins than the corresponding controls. The data are shown as the mean ± SD. *P < 0.05. The scale bar represents 50 μm.

liferation and differentiation of PDLSC by paracrine effects, but the mechanisms need further study.

Although stem cell sheets, a cell delivery strategy that maintains contact among cells, have been widely used in periodontal tissue engineering research, there is no ideal method with which to obtain enough cell sheets to meet the needs of clinical application [40, 43] . Until now, cell sheet formation was a long and complicated process [44, 45]. Although Vc induction and osteogenic induction are both common ways to produce cell sheets, there is no consensus on which method is better for periodontal tissue regeneration [44, 56]. USCs cocultured with PDLSCs at three ratios promoted PDLSC sheets formation, resulting in more cell layers and ECM production and promoting the differentiation capacity of osteogenic matrix cell sheets in vitro (**Figures 6**, **7**). Moreover, we found that cell sheets produced at a PDLSC/USC ratio of 1/2 distinctly increased the tissue formation of PDLSC sheets in vivo (**Figure 8**). These results demonstrate that USCs facilitate the formation and multiple dif-

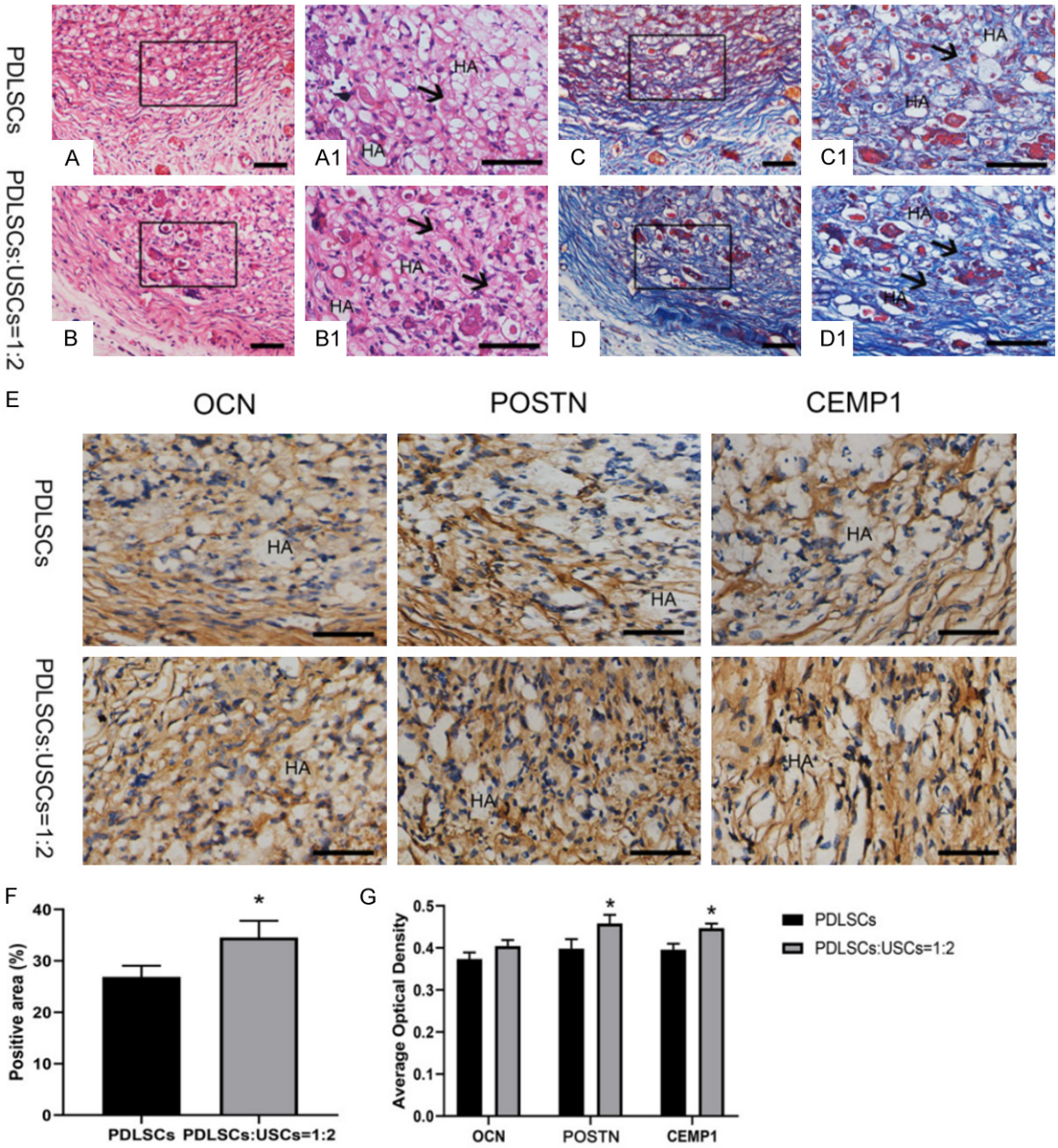

Figure 8. Regeneration of new tissue by PDLSC sheets mixed with hydroxyapatite in nude mice. A, A1, B, B1. Representative H&E staining images of the transplants showing regenerated tissues in the corresponding control group were few in number and scattered. C, C1, D, D1. Representative Masson's trichrome staining images of the transplants showed that a quantity of dense and notable collagenous fibers had formed. E. Representative immunohistochemical staining images of the transplants showed that both groups positively expressed OCN, POSTN and CEMP1. F. Quantitative analysis of the new hard tissue area. G. The quantitation of OCN, POSTN and CEMP1 immunohistochemical staining showed that the expression levels of CEMP1 and POSTN were higher in the experimental group than in the control groups. The black arrow shows regenerated tissue. The data are shown as the mean ± SD. *P < 0.05. The scale bar represents 50 μm.

ferentiation of PDLSC sheets in osteogenic medium through noncontact coculture and that the osteogenic matrix PDLSC sheets regenerate tissue-expressed cementogenic and osteogenic genes in vivo. Therefore, the noncontact coculture of osteogenic matrix PDLSC sheets with USCs is a potential method of periodontal tissue engineering.

Coculture is a powerful tool with which to understand the interactions between cells. Both cell-to-cell contact and paracrine mechanisms play

important roles in coculture systems [54]. Although a noncontact coculture system cannot completely simulate the spatial relationship between two types of cells, it allows for the real-time observation of the effects of USCs on PDLSCs and avoids the use of difficult cellular and molecular analyses in a direct coculture system [57]. USCs exhibit a weaker capacity for osteogenesis as seed cells than bone marrow-derived MSCs, and USCs are not the best seed cells for periodontal regeneration; however, the paracrine effect of USCs has attracted wide attention [38]. Noncontact coculture with USCs at three ratios clearly increased the osteoblastic/cementoblastic differentiation of PDLSCs compared with PDLSCs cultured alone, which suggests the beneficial use of USCs as an auxiliary factor with the potential to facilitate PDLSC regeneration to alveolar bone and cementum for periodontal tissue regeneration.

In this study, we confirmed that USCs can promote the osteogenic and cementogenic differentiation and proliferation of PDLSCs, accelerate cell sheet formation, and increase the protein expression of osteogenic and cementogenic genes in PDLSCs through noncontact coculture; furthermore, these effects were shown to be ratio-dependent. Osteogenic matrix PDLSC sheets that are noncontact cocultured with USCs are a potential way to regenerate cementum/bone-like structures in vivo. These results suggest the use of USCs in periodontal tissue engineering through their paracrine effect on PDLSCs for the first time. Although the molecular mechanism of the effect of noncontact coculture with USCs on PDLSCs requires further study, the noninvasive collection method of USCs and their stable expansion make USCs a prospective new strategy for application in clinical periodontal tissue repair.

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Disclosure of conflict of interest

None.

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References

[1] Pihlstrom BL, Michalowicz BS and Johnson NW. Periodontal diseases. Lancet 2005; 366: 1809-1820.

[2] Cortelli SC, Cortelli JR, Romeiro RL, Costa FO, Aquino DR, Orzechowski PR, Araujo VC and Duarte PM. Frequency of periodontal pathogens in equivalent peri-implant and periodontal clinical statuses. Arch Oral Biol 2013; 58: 67-74.

[3] Chen FM, Zhang J, Zhang M, An Y, Chen F and Wu ZF. A review on endogenous regenerative technology in periodontal regenerative medicine. Biomaterials 2010; 31: 7892-7927.

[4] Ramseier CA, Rasperini G, Batia S and Giannobile WV. Advanced reconstructive technologies for periodontal tissue repair. Periodontol 2000 2012; 59: 185-202.

[5] Smith PC, Martinez C, Caceres M and Martinez J. Research on growth factors in periodontology. Periodontol 2000 2015; 67: 234-250.

[6] Susin C and Wikesjo UM. Regenerative periodontal therapy: 30 years of lessons learned and unlearned. Periodontol 2000 2013; 62: 232-242.

[7] Hynes K, Menicanin D, Gronthos S and Bartold PM. Clinical utility of stem cells for periodontal regeneration. Periodontol 2000 2012; 59: 203-227.

[8] Xu XY, Li X, Wang J, He XT, Sun HH and Chen FM. Concise review: periodontal tissue regeneration using stem cells: strategies and translational considerations. Stem Cells Transl Med 2019; 8: 392-403.

[9] Feng F, Akiyama K, Liu Y, Yamaza T, Wang TM, Chen JH, Wang BB, Huang GT, Wang S and Shi S. Utility of PDL progenitors for in vivo tissue regeneration: a report of 3 cases. Oral Dis 2010; 16: 20-28.

[10] Tsumanuma Y, Iwata T, Washio K, Yoshida T, Yamada A, Takagi R, Ohno T, Lin K, Yamato M, Ishikawa I, Okano T and Izumi Y. Comparison of different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model. Biomaterials 2011; 32: 5819-5825.

[11] Simsek SB, Keles GC, Baris S and Cetinkaya BO. Comparison of mesenchymal stem cells and autogenous cortical bone graft in the

treatment of class II furcation defects in dogs. Clin Oral Investig 2012; 16: 251-258.
[12] Aimetti M, Ferrarotti F, Cricenti L, Mariani GM and Romano F. Autologous dental pulp stem cells in periodontal regeneration: a case report. Int J Periodontics Restorative Dent 2014; 34 Suppl 3: s27-33.
[13] Tassi SA, Sergio NZ, Misawa MYO and Villar CC. Efficacy of stem cells on periodontal regeneration: systematic review of pre-clinical studies. J Periodontal Res 2017; 52: 793-812.
[14] Hernandez-Monjaraz B, Santiago-Osorio E, Ledesma-Martinez E, Alcauter-Zavala A and Mendoza-Nunez VM. Retrieval of a periodontally compromised tooth by allogeneic grafting of mesenchymal stem cells from dental pulp: a case report. J Int Med Res 2018; 46: 2983-2993.
[15] Park JY, Jeon SH and Choung PH. Efficacy of periodontal stem cell transplantation in the treatment of advanced periodontitis. Cell Transplant 2011; 20: 271-285.
[16] Bright R, Hynes K, Gronthos S and Bartold PM. Periodontal ligament-derived cells for periodontal regeneration in animal models: a systematic review. J Periodontal Res 2015; 50: 160-172.
[17] Cha Y, Jeon M, Lee HS, Kim S, Kim SO, Lee JH and Song JS. Effects of in vitro osteogenic induction on in vivo tissue regeneration by dental pulp and periodontal ligament stem cells. J Endod 2015; 41: 1462-1468.
[18] Panduwawala CP, Zhan X, Dissanayaka WL, Samaranayake LP, Jin L and Zhang C. In vivo periodontal tissue regeneration by periodontal ligament stem cells and endothelial cells in three-dimensional cell sheet constructs. J Periodontal Res 2017; 52: 408-418.
[19] Santamaria S, Sanchez N, Sanz M and Garcia-Sanz JA. Comparison of periodontal ligament and gingiva-derived mesenchymal stem cells for regenerative therapies. Clin Oral Investig 2017; 21: 1095-1102.
[20] Han J, Menicanin D, Gronthos S and Bartold PM. Stem cells, tissue engineering and periodontal regeneration. Aust Dent J 2014; 59 Suppl 1: 117-130.
[21] Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY and Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 2004; 364: 149-155.
[22] Lee JH, Um S, Jang JH and Seo BM. Effects of VEGF and FGF-2 on proliferation and differentiation of human periodontal ligament stem cells. Cell Tissue Res 2012; 348: 475-484.
[23] Teramatsu Y, Maeda H, Sugii H, Tomokiyo A, Hamano S, Wada N, Yuda A, Yamamoto N, Koori K and Akamine A. Expression and effects of epidermal growth factor on human periodontal ligament cells. Cell Tissue Res 2014; 357: 633-643.
[24] Sawada K, Takedachi M, Yamamoto S, Morimoto C, Ozasa M, Iwayama T, Lee CM, Okura H, Matsuyama A, Kitamura M and Murakami S. Trophic factors from adipose tissue-derived multi-lineage progenitor cells promote cytodifferentiation of periodontal ligament cells. Biochem Biophys Res Commun 2015; 464: 299-305.
[25] Wu Z, Dai W, Wang P, Zhang X, Tang Y, Liu L, Wang Q, Li M and Tang C. Periostin promotes migration, proliferation, and differentiation of human periodontal ligament mesenchymal stem cells. Connect Tissue Res 2018; 59: 108-119.
[26] Wu Y, Cao H, Yang Y, Zhou Y, Gu Y, Zhao X, Zhang Y, Zhao Z, Zhang L and Yin J. Effects of vascular endothelial cells on osteogenic differentiation of noncontact co-cultured periodontal ligament stem cells under hypoxia. J Periodontal Res 2013; 48: 52-65.
[27] Yu M, Wang L, Ba P, Li L, Sun L, Duan X, Yang P, Yang C and Sun Q. Osteoblast progenitors enhance osteogenic differentiation of periodontal ligament stem cells. J Periodontol 2017; 88: e159-e168.
[28] Zhang H, Liu S, Zhu B, Xu Q, Ding Y and Jin Y. Composite cell sheet for periodontal regeneration: crosstalk between different types of MSCs in cell sheet facilitates complex periodontal-like tissue regeneration. Stem Cell Res Ther 2016; 7: 168.
[29] Mitchell AC, Briquez PS, Hubbell JA and Cochran JR. Engineering growth factors for regenerative medicine applications. Acta Biomater 2016; 30: 1-12.
[30] Zhang Y, McNeill E, Tian H, Soker S, Andersson KE, Yoo JJ and Atala A. Urine derived cells are a potential source for urological tissue reconstruction. J Urol 2008; 180: 2226-2233.
[31] Kang HS, Choi SH, Kim BS, Choi JY, Park GB, Kwon TG and Chun SY. Advanced properties of urine derived stem cells compared to adipose tissue derived stem cells in terms of cell proliferation, immune modulation and multi differentiation. J Korean Med Sci 2015; 30: 1764-1776.
[32] Gao P, Jiang D, Liu W, Li H and Li Z. Urine-derived stem cells, a new source of seed cells for tissue engineering. Curr Stem Cell Res Ther 2016; 11: 547-553.
[33] Wu S, Liu Y, Bharadwaj S, Atala A and Zhang Y. Human urine-derived stem cells seeded in a modified 3D porous small intestinal submuco-

sa scaffold for urethral tissue engineering. Biomaterials 2011; 32: 1317-1326.
[34] Bharadwaj S, Liu G, Shi Y, Wu R, Yang B, He T, Fan Y, Lu X, Zhou X, Liu H, Atala A, Rohozinski J and Zhang Y. Multipotential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology. Stem Cells 2013; 31: 1840-1856.
[35] Fu Y, Guan J, Guo S, Guo F, Niu X, Liu Q, Zhang C, Nie H and Wang Y. Human urine-derived stem cells in combination with polycaprolactone/gelatin nanofibrous membranes enhance wound healing by promoting angiogenesis. J Transl Med 2014; 12: 274.
[36] Guan JJ, Niu X, Gong FX, Hu B, Guo SC, Lou YL, Zhang CQ, Deng ZF and Wang Y. Biological characteristics of human-urine-derived stem cells: potential for cell-based therapy in neurology. Tissue Eng Part A 2014; 20: 1794-1806.
[37] Wu C, Chen L, Huang YZ, Huang Y, Parolini O, Zhong Q, Tian X and Deng L. Comparison of the proliferation and differentiation potential of human urine-, placenta decidua basalis-, and bone marrow-derived stem cells. Stem Cells Int 2018; 2018: 7131532.
[38] Pei M, Li J, Zhang Y, Liu G, Wei L and Zhang Y. Expansion on a matrix deposited by nonchondrogenic urine stem cells strengthens the chondrogenic capacity of repeated-passage bone marrow stromal cells. Cell Tissue Res 2014; 356: 391-403.
[39] Zhu Q, Li Q, Niu X, Zhang G, Ling X, Zhang J, Wang Y and Deng Z. Extracellular vesicles secreted by human urine-derived stem cells promote ischemia repair in a mouse model of hind-limb ischemia. Cell Physiol Biochem 2018; 47: 1181-1192.
[40] Chen FM, Sun HH, Lu H and Yu Q. Stem cell-delivery therapeutics for periodontal tissue regeneration. Biomaterials 2012; 33: 6320-6344.
[41] Kelm JM and Fussenegger M. Scaffold-free cell delivery for use in regenerative medicine. Adv Drug Deliv Rev 2010; 62: 753-764.
[42] Baldari S, Di Rocco G, Piccoli M, Pozzobon M, Muraca M and Toietta G. Challenges and strategies for improving the regenerative effects of mesenchymal stromal cell-based therapies. Int J Mol Sci 2017; 18.
[43] Iwata T, Washio K, Yoshida T, Ishikawa I, Ando T, Yamato M and Okano T. Cell sheet engineering and its application for periodontal regeneration. J Tissue Eng Regen Med 2015; 9: 343-356.
[44] Wei F, Qu C, Song T, Ding G, Fan Z, Liu D, Liu Y, Zhang C, Shi S and Wang S. Vitamin C treatment promotes mesenchymal stem cell sheet formation and tissue regeneration by elevating telomerase activity. J Cell Physiol 2012; 227: 3216-3224.
[45] Ueha T, Akahane M, Shimizu T, Uchihara Y, Morita Y, Nitta N, Kido A, Inagaki Y, Kawate K and Tanaka Y. Utility of tricalcium phosphate and osteogenic matrix cell sheet constructs for bone defect reconstruction. World J Stem Cells 2015; 7: 873-882.
[46] Onishi T, Shimizu T, Akahane M, Omokawa S, Okuda A, Kira T, Inagak Y and Tanaka Y. Osteogenic extracellular matrix sheet for bone tissue regeneration. Eur Cell Mater 2018; 36: 68-80.
[47] Arzate H, Zeichner-David M and Mercado-Celis G. Cementum proteins: role in cementogenesis, biomineralization, periodontium formation and regeneration. Periodontol 2000 2015; 67: 211-233.
[48] Alvarez-Perez MA, Narayanan S, Zeichner-David M, Rodriguez Carmona B and Arzate H. Molecular cloning, expression and immunolocalization of a novel human cementum-derived protein (CP-23). Bone 2006; 38: 409-419.
[49] Lynch SE, de Castilla GR, Williams RC, Kiritsy CP, Howell TH, Reddy MS and Antoniades HN. The effects of short-term application of a combination of platelet-derived and insulin-like growth factors on periodontal wound healing. J Periodontol 1991; 62: 458-467.
[50] Gotz W, Kunert D, Zhang D, Kawarizadeh A, Lossdorfer S and Jager A. Insulin-like growth factor system components in the periodontium during tooth root resorption and early repair processes in the rat. Eur J Oral Sci 2006; 114: 318-327.
[51] Stavropoulos A and Wikesjo UM. Growth and differentiation factors for periodontal regeneration: a review on factors with clinical testing. J Periodontal Res 2012; 47: 545-553.
[52] Miron RJ, Wei L, Yang S, Caluseru OM, Sculean A and Zhang Y. Effect of enamel matrix derivative on periodontal wound healing and regeneration in an osteoporotic model. J Periodontol 2014; 85: 1603-1611.
[53] Gauthier P, Yu Z, Tran QT, Bhatti FU, Zhu X and Huang GT. Cementogenic genes in human periodontal ligament stem cells are downregulated in response to osteogenic stimulation while upregulated by vitamin C treatment. Cell Tissue Res 2017; 368: 79-92.
[54] Paschos NK, Brown WE, Eswaramoorthy R, Hu JC and Athanasiou KA. Advances in tissue engineering through stem cell-based co-culture. J Tissue Eng Regen Med 2015; 9: 488-503.
[55] Szpalski C, Sagebin F, Barbaro M and Warren SM. The influence of environmental factors on

bone tissue engineering. J Biomed Mater Res B Appl Biomater 2013; 101: 663-675.

[56] Akahane M, Nakamura A, Ohgushi H, Shigematsu H, Dohi Y and Takakura Y. Osteogenic matrix sheet-cell transplantation using osteoblastic cell sheet resulted in bone formation without scaffold at an ectopic site. J Tissue Eng Regen Med 2008; 2: 196-201.

[57] Clarkin CE, Emery RJ, Pitsillides AA and Wheeler-Jones CP. Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-mediated crosstalk to endothelial cells. J Cell Physiol 2008; 214: 537-544.